

# *Acta Medica Okayama*

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*Volume 53, Issue 1*

1999

*Article 4*

FEBRUARY 1999

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## Formation of gamma-glutamylpropargylglycylglycine from propargylglycine in human blood and erythrocytes

Noboru Nagamine\*

Jun Ohta<sup>†</sup>

Noriyoshi Masuoka<sup>‡</sup>

Hiroyuki Kodama\*\*

Toshihiko Ubuka<sup>††</sup>

\*Okayama University,

<sup>†</sup>Okayama University,

<sup>‡</sup>Okayama University,

\*\*Kochi Medical School,

<sup>††</sup>Okayama University,

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Noboru Nagamine, Jun Ohta, Noriyoshi Masuoka, Hiroyuki Kodama, and  
Toshihiko Ubuka

## Abstract

Gamma-Glutamylpropargylglycylglycine (gamma-Glu-PPG-Gly) was isolated as a metabolite of propargylglycine (2-amino-4-pentynoic acid, a natural and synthetic inhibitor of cystathionine gamma-lyase) from human blood incubated with D,L-propargylglycine in the presence of L-glutamate and glycine, and identified by fast-atom-bombardment mass spectrometry, indicating that human blood can metabolize propargylglycine to gamma-Glu-PPG-Gly. When whole blood was incubated with 2 mM D,L-propargylglycine in the presence of 10 mM L-glutamate and 10 mM glycine at 37 degrees C for 16h, 0.094 $\pm$ 0.013 micromol of gamma-Glu-PPG-Gly was formed per ml of whole blood. When erythrocytes were incubated under the same conditions for 16h, 0.323 $\pm$ 0.060 micromol of gamma-Glu-PPG-Gly was formed per ml of erythrocytes, suggesting a large contribution of erythrocytes to gamma-Glu-PPG-Gly formation in whole blood. The apparent  $K_m$  value of gamma-Glu-PPG-Gly formation in human erythrocytes for D,L-propargylglycine was 0.32 mM. The observed rate of gamma-Glu-PPG-Gly formation and the  $K_m$  value for D,L-propargylglycine suggest that metabolism of propargylglycine to gamma-Glu-PPG-Gly can play a definite biological role in human subjects who are loaded with propargylglycine.

**KEYWORDS:** propargylglycine, glutathione analogue, 2-amino-4-pentynoic acid, cystathionine gamma-lyase

## Formation of $\gamma$ -Glutamylpropargylglycylglycine from Propargylglycine in Human Blood and Erythrocytes

Noboru NAGAMINE<sup>a</sup>, Jun OHTA<sup>a\*</sup>, Noriyoshi MASUOKA<sup>a</sup>, Hiroyuki KODAMA<sup>b</sup> and Toshihiko UBUKA<sup>a</sup>

<sup>a</sup>Department of Biochemistry, Okayama University Medical School, Okayama 700-8558 and <sup>b</sup>Department of Chemistry, Kochi Medical School, Nankoku, Kochi 783-8505, Japan

$\gamma$ -Glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) was isolated as a metabolite of propargylglycine (2-amino-4-pentynoic acid, a natural and synthetic inhibitor of cystathionine  $\gamma$ -lyase) from human blood incubated with D,L-propargylglycine in the presence of L-glutamate and glycine, and identified by fast-atom-bombardment mass spectrometry, indicating that human blood can metabolize propargylglycine to  $\gamma$ -Glu-PPG-Gly. When whole blood was incubated with 2mM D,L-propargylglycine in the presence of 10mM L-glutamate and 10mM glycine at 37°C for 16h,  $0.094 \pm 0.013 \mu\text{mol}$  of  $\gamma$ -Glu-PPG-Gly was formed per ml of whole blood. When erythrocytes were incubated under the same conditions for 16h,  $0.323 \pm 0.060 \mu\text{mol}$  of  $\gamma$ -Glu-PPG-Gly was formed per ml of erythrocytes, suggesting a large contribution of erythrocytes to  $\gamma$ -Glu-PPG-Gly formation in whole blood. The apparent  $K_m$  value of  $\gamma$ -Glu-PPG-Gly formation in human erythrocytes for D,L-propargylglycine was 0.32mM. The observed rate of  $\gamma$ -Glu-PPG-Gly formation and the  $K_m$  value for D,L-propargylglycine suggest that metabolism of propargylglycine to  $\gamma$ -Glu-PPG-Gly can play a definite biological role in human subjects who are loaded with propargylglycine.

**Key words:** propargylglycine, glutathione analogue, 2-amino-4-pentynoic acid, cystathionine  $\gamma$ -lyase

**P**ropargylglycine (2-amino-4-pentynoic acid) is an acetylenic amino acid, which was initially chemically synthesized as an inhibitor of microbial growth (1) and, later, was isolated as a streptomycete fermentation product (2), as a constituent of *Amanita pseudoporphyria*

(3) and as a toxic component of *Amanita abrupta* which induces a decrease of liver glycogen in mice (4, 5).

Abeles and Walsh found that propargylglycine inactivates cystathionine  $\gamma$ -lyase (EC 4.4.1.1, L-cystathionine cysteine-lyase (deaminating)) (6), the enzyme responsible for cysteine formation from cystathionine in the transsulfurative metabolism of L-methionine in mammalian tissues. Thus, propargylglycine has been used for studies of sulfur amino acid metabolism such as unusual cystathionine metabolism in cystathioninuria, cystathionine  $\gamma$ -lyase deficiency (7-9), and utilization of methionine sulfur as a source of the sulfur in glutathione (10-14) and metallothionein (12, 14). Further, a possible use of propargylglycine for cysteine depletion in cancer therapy has been suggested, since certain malignant cell lines require L-cyst(e)ine as an essential nutrient (15, 16). It has also been reported that propargylglycine diminishes metallothionein-mediated cisplatin resistance of human and mouse bladder tumors inoculated in nude mice (17, 18).

We identified *N*-acetylpropargylglycine (19) and  $\gamma$ -glutamylpropargylglycylglycine (*N*-[*N*- $\gamma$ -glutamyl(propargylglycyl)]glycine,  $\gamma$ -Glu-PPG-Gly) (20, 21) as metabolites of propargylglycine in rats given propargylglycine. It is likely that the *in vivo* effect of propargylglycine could be modulated through conversion of propargylglycine to these metabolites of propargylglycine. We are interested in propargylglycine metabolism in human tissues, since propargylglycine is a naturally occurring biologically active compound and a potentially therapeutic agent as discussed above. In the present study, we demonstrated  $\gamma$ -Glu-PPG-Gly formation from propargylglycine in human blood, and investigated  $\gamma$ -Glu-PPG-Gly formation in human erythrocytes.

\* To whom correspondence should be addressed.

## Materials and Methods

**Materials.** Human venous blood was collected in heparinized tubes from normal human volunteers (31 to 50 years old). Human erythrocytes were separated by centrifugation of the blood at  $1,000 \times g$  for 5 min, and washed three times with 2 volumes of Hanks medium (22) (pH 7.4, in air). D,L-Propargylglycine was obtained from Sigma Chemical Co., St. Louis, MO, USA. Monosodium L-glutamate and glycine were from Wako Pure Chemical Ind., Ltd., Osaka, Japan. Dowex 50W-X8 (200–400 mesh) and Dowex 1-X4 (200–400 mesh) were from Bio-Rad, Richmond, CA, USA. FUNACEL, microcrystalline cellulose was purchased from Funakoshi Co., Ltd., Tokyo, Japan. The  $\gamma$ -Glu-PPG-Gly isolated from the livers of D,L-propargylglycine-administered rats was used as authentic  $\gamma$ -Glu-PPG-Gly (21).

**Incubations.** The incubation mixture was a mixture of 3 volumes of blood or suspension of erythrocytes in Hanks medium (pH 7.4, in air) and one volume of substrate mixture, which was prepared as a mixture of the following 4 solutions: 300 mM D,L-propargylglycine, 150 mM monosodium L-glutamate, 300 mM glycine, and 150 mM NaCl.

Unless otherwise mentioned, incubations were conducted at 37°C with 0.8 ml of incubation mixture in sealed 15 ml glass tubes. The incubation mixture was maintained in suspension during the incubation period by shaking the 15 ml tube at 130 strokes per min. At the times indicated, 0.7 ml aliquot of the incubation mixture was added directly to 0.14 ml of 2 M acetic acid. The resulting hemolysate was mixed with 0.84 ml of 6 % (w/v) sulfosalicylic acid and centrifuged at  $10,000 \times g$  for 10 min at 4°C. 0.5 ml of the supernatant was analyzed by amino acid analysis as described below. Incubations without D,L-propargylglycine served as blanks, and values for incubations without D,L-propargylglycine were subtracted from the values for the corresponding incubations with D,L-propargylglycine to calculate the amount of  $\gamma$ -Glu-PPG-Gly formed during the incubation because a compound with almost the same elution volume on amino acid analysis as  $\gamma$ -Glu-PPG-Gly was formed in most of the blank incubations without D,L-propargylglycine. The peak area of the compound formed in the blank incubations of whole blood in the presence of 10 or 20 mM each of glutamate and glycine was 16–34 % of that of the compound plus  $\gamma$ -Glu-PPG-Gly formed in the corresponding incubations

with D,L-propargylglycine, whereas the peak area of the compound formed in the blank incubations of washed erythrocytes corresponded to 0–0.03  $\mu$ mol of  $\gamma$ -Glu-PPG-Gly/ml of erythrocytes/16 h. Neither  $\gamma$ -Glu-PPG-Gly nor the compound which overlaps  $\gamma$ -Glu-PPG-Gly on amino acid analysis were detected in human blood incubated without any substrates for 16 h.

In the experiment to study the distribution of  $\gamma$ -Glu-PPG-Gly formed in erythrocytes between incubation medium and erythrocytes, 2 incubations were conducted for each erythrocyte preparation, and the  $\gamma$ -Glu-PPG-Gly content of the incubation mixture and that of the incubation medium were determined. For the determination of  $\gamma$ -Glu-PPG-Gly content of the incubation medium, the incubation mixture was centrifuged at  $1,000 \times g$  for 5 min at 4°C. The incubation medium obtained as the supernatant was mixed with one volume of 6 % (w/v) sulfosalicylic acid and centrifuged at  $10,000 \times g$  for 10 min at 4°C. The resulting supernatant was analyzed by amino acid analysis.

Several incubations were conducted with 28 ml of incubation mixture containing 21 ml of whole blood, 2 mM D,L-propargylglycine, 20 mM L-glutamate, and 20 mM glycine in sealed 50 ml plastic tubes for the purpose of yielding enough  $\gamma$ -Glu-PPG-Gly for the subsequent isolation and identification of the compound. These incubations were carried out at 37°C for 16 h while the incubation tubes were slowly rotated to maintain the cells in suspension. Then, the incubation mixture was centrifuged at  $1,000 \times g$  for 5 min. The supernatant containing the plasma was removed, and the resulting blood cells were stored at –20°C until use.

**Isolation of  $\gamma$ -Glu-PPG-Gly from human blood incubated with propargylglycine.** Blood cells, 53 g, were obtained from 84 ml of human blood incubated with 2 mM D,L-propargylglycine in the presence of 20 mM L-glutamate and 20 mM glycine for 16 h as described above, and were mixed well with 2 volumes of 10 % (w/v) trichloroacetic acid and one volume of water. The mixture obtained was sonicated at 20 kHz for 10 min at 0°C and centrifuged at  $2,000 \times g$  for 10 min. The resulting pellet was resuspended in 106 ml of 5 % (w/v) trichloroacetic acid and centrifuged as above. The supernatants obtained from these 2 centrifugations were combined, filtered through Whatman No. 1 paper, and then applied to a column of Dowex 50W-X8 (200–400 mesh, H<sup>+</sup>-form, 4.2 cm  $\times$  7.2 cm). The column was washed with 500 ml of water and then eluted with 500 ml

of 2M  $\text{NH}_3$ . The eluate was dried under reduced pressure at 40°C. The resulting residue was dissolved in 48 ml of water. The solution (pH 7) was applied to a column of Dowex 1-X4 (200–400 mesh, acetate form, 1.5 cm  $\times$  5.7 cm). The column was washed with 100 ml of water and then eluted with 160 ml of 0.5M acetic acid with 3 ml portions being collected. The eluate between 54 ml and 84 ml was dried as above, and dissolved in 2 ml of water. The solution, which contained Asp as well as  $\gamma$ -Glu-PPG-Gly, was adjusted to pH 7 with 2M  $\text{NH}_3$ , mixed with 10  $\mu$ l of 6M  $\text{AgNO}_3$ , and left at 0°C for 10 min. The resulting precipitate, which presumably is the silver acetylide of  $\gamma$ -Glu-PPG-Gly, was collected by centrifugation, washed with 1 ml of ice cold water, and resuspended in 1 ml of 0.06M HCl. The suspension was left at 0°C for 10 min and centrifuged for removal of the AgCl formed. The supernatant was applied to a column of Dowex 50W-X8 (200–400 mesh,  $\text{H}^+$ -form, 0.7 cm  $\times$  2.5 cm). The column was washed with 5 ml of water and then eluted with 5 ml of 2M  $\text{NH}_3$ . The eluate was dried as above and used for its identification. In the above isolation procedure, presence and purity of  $\gamma$ -Glu-PPG-Gly in each fraction were checked by amino acid analysis.

**Acid hydrolysis, carboxypeptidase reaction, and  $\gamma$ -glutamyltranspeptidase reaction of  $\gamma$ -Glu-PPG-Gly fraction.** Acid hydrolysis, carboxypeptidase reaction, and  $\gamma$ -glutamyltranspeptidase reaction of the  $\gamma$ -Glu-PPG-Gly fraction were conducted as previously described (21).

**Fast-atom-bombardment mass spectrometry.** Fast-atom-bombardment (FAB) mass spectrometry was carried out on a Shimadzu 9020-DF gas chromatograph-mass spectrometer equipped with a Shimadzu SCAP 1123 data system (Central Research Laboratory, Okayama University Medical School). The target surface was bombarded by a beam of energetic argon atoms at 5 keV; glycerol was used as a matrix.

**Amino acid analysis.** Amino acid analysis was conducted with a Hitachi KLA-5 amino acid analyzer using a column of custom ion-exchange resin No. 2613, 0.9 cm  $\times$  55 cm. Chromatography was performed with 0.2N sodium citrate buffer containing 8% ethanol (pH 3.02) at a flow rate of 1 ml per min at 55°C (21).

**Thin layer chromatography and high-voltage paper electrophoresis.** Thin layer chromatography was performed on a thin layer of FUNACEL, microcrystalline cellulose, using a solvent system of n-butanol-acetic acid-water (4:1:1, by volume) (21). High-

voltage paper electrophoresis was carried out using 95% pyridine-acetic acid-water (0.5:10.0:89.5, by volume; pH 3.1) as a buffer solution (23) on Whatman No. 1 paper at 3,000 V for 30 min (21). One percent (w/v) ninhydrin–2% (v/v) pyridine in acetone was used for color development.

**Statistics.** Data obtained by the present study were analyzed with the Student's *t*-test or analysis of variance followed by comparison of means with Tukey's  $\omega$ -procedure.

## Results and Discussion

**Isolation of  $\gamma$ -Glu-PPG-Gly from human blood incubated with propargylglycine and its identification.** In order to show that  $\gamma$ -Glu-PPG-Gly can be formed from propargylglycine in human blood, we did the following: We attempted to isolate  $\gamma$ -Glu-PPG-Gly from human blood incubated with 2 mM D,L-propargylglycine in the presence of 20 mM L-glutamate and 20 mM glycine for 16 h by ion-exchange chromatography (21) and precipitation of the silver acetylide of  $\gamma$ -Glu-PPG-Gly, and obtained  $\gamma$ -Glu-PPG-Gly preparation equivalent to 11.4  $\mu$ mol of  $\gamma$ -Glu-PPG-Gly from 84 ml of the human blood with a recovery rate from the blood cell extract of 83%.

The  $\gamma$ -Glu-PPG-Gly preparation from human blood showed a single peak, a single spot, and a single band, when analyzed by amino acid analysis, thin layer chromatography, and high-voltage paper electrophoresis, respectively, which coincided to those of authentic  $\gamma$ -Glu-PPG-Gly.

As shown in Fig. 1, the fast-atom-bombardment mass spectrum of the  $\gamma$ -Glu-PPG-Gly preparation from human blood was essentially the same as that of authentic  $\gamma$ -Glu-PPG-Gly, and the signals in the spectrum were assigned successfully as previously described (21):  $m/z$  300 [ $\text{MH}^+$  (molecular ion plus proton)], 225 [ $\text{MH}^+ - (\text{H}_2\text{N}-\text{CH}_2-\text{CO}_2\text{H})$ ], 171 [ $\text{MH}^+ - (\text{HN}-\text{CH}(\text{CO}_2\text{H})-\text{CH}_2-\text{CH}_2-\text{CO})$ ], and 130 [ $(\text{HN}-\text{CH}(\text{CO}_2\text{H})-\text{CH}_2-\text{CH}_2-\text{CO}) \text{H}^+$ ]. When the  $\gamma$ -Glu-PPG-Gly preparation from human blood was hydrolyzed in 6M HCl at 110°C for 4 h, it was completely degraded to form propargylglycine, glutamate, and glycine at a molar ratio of 0.44:1.00:1.00, as reported for authentic  $\gamma$ -Glu-PPG-Gly (21). Glycine and glutamate were released by carboxypeptidase reaction and  $\gamma$ -glutamyltranspeptidase reaction of the  $\gamma$ -Glu-PPG-Gly preparation, respectively (data not

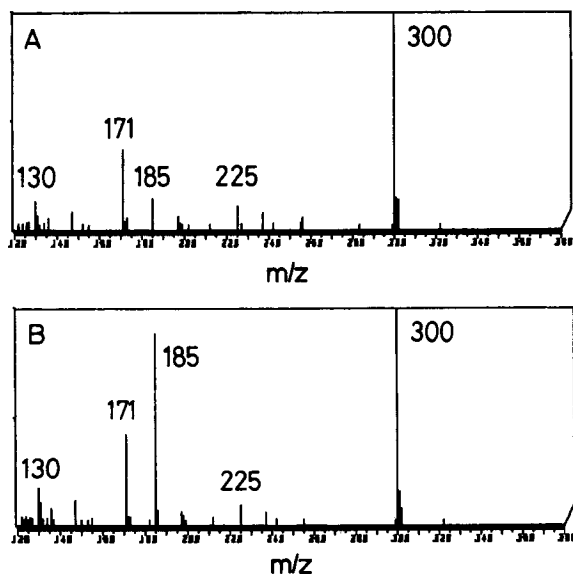


Fig. 1 Fast-atom-bombardment mass spectra of  $\gamma$ -glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) isolated from human blood incubated with 2 mM D,L-propargylglycine in the presence of 10 mM L-glutamate and 10 mM glycine (A) and authentic  $\gamma$ -Glu-PPG-Gly (B). The signal at  $m/z$  185 is due to glycerol used as a matrix.

shown), supporting the existence of C-terminal glycine residue and  $\gamma$ -glutamyl moiety in the isolated compound.

These results show that  $\gamma$ -Glu-PPG-Gly was definitely isolated from the human blood incubated with propargylglycine, showing that  $\gamma$ -Glu-PPG-Gly can be formed from propargylglycine in human blood.

**$\gamma$ -Glu-PPG-Gly formation from propargylglycine in human blood.** As shown in Table 1, when human blood was incubated with 2 mM D,L-propargylglycine in the presence of 0–20 mM each of L-glutamate and glycine for 16 h, we observed significant  $\gamma$ -Glu-PPG-Gly formation ( $P < 0.01$ , by the Student's *t*-test). Addition of L-glutamate and glycine to the incubation medium resulted in a significant increase of  $\gamma$ -Glu-PPG-Gly formed, showing that  $\gamma$ -Glu-PPG-Gly formation from propargylglycine in human blood is dependent on L-glutamate or glycine availability.

**$\gamma$ -Glu-PPG-Gly formation from propargylglycine in human erythrocytes.** Erythrocytes are the major cell population of human blood. Therefore, we investigated  $\gamma$ -Glu-PPG-Gly formation from propargylglycine in human erythrocytes. When human erythrocytes were incubated with 2 mM D,L-propargylglycine in the presence of 10 mM L-glutamate and 10 mM glycine,

we observed  $\gamma$ -Glu-PPG-Gly formation in proportion to the incubation time and to the amount of erythrocytes as shown in Figs. 2 and 3. Under these incubation conditions,  $\gamma$ -Glu-PPG-Gly formed in human erythrocytes was  $0.323 \pm 0.060 \mu\text{mol/ml}$  of erythrocytes/16 h ( $n = 12$ ). This rate of  $\gamma$ -Glu-PPG-Gly formation in human erythrocytes was high enough to account for the  $\gamma$ -Glu-PPG-Gly formation observed in human whole blood, suggesting a large contribution of erythrocytes to  $\gamma$ -Glu-PPG-Gly formation in whole blood.

As shown in Table 2, most of  $\gamma$ -Glu-PPG-Gly formed was retained inside the erythrocytes. When frozen and thawed erythrocytes were used instead of intact erythrocytes for incubations,  $\gamma$ -Glu-PPG-Gly formation was negligible (Table 3), suggesting that continuous ATP formation may be required for  $\gamma$ -Glu-PPG-Gly synthesis.

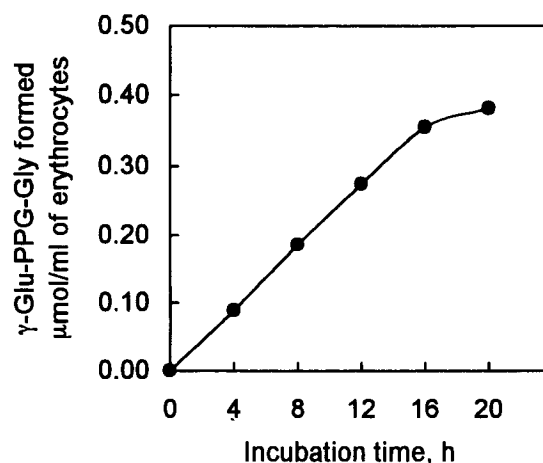
Table 4 shows the effect of extracellular glutamate and glycine on  $\gamma$ -Glu-PPG-Gly formation from D,L-propargylglycine in erythrocytes. The rate of  $\gamma$ -Glu-PPG-Gly formation for substrate mixture without glutamate or glycine was significantly lower than that for substrate mixture containing both glutamate and glycine, showing that  $\gamma$ -Glu-PPG-Gly formation from propargylglycine in human erythrocytes is dependent on the availability of both glutamate and glycine. It is likely that, in cells containing high levels of glutamate and glycine, propargyl-

Table 1  $\gamma$ -Glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) formation from D,L-propargylglycine in human blood<sup>a</sup>

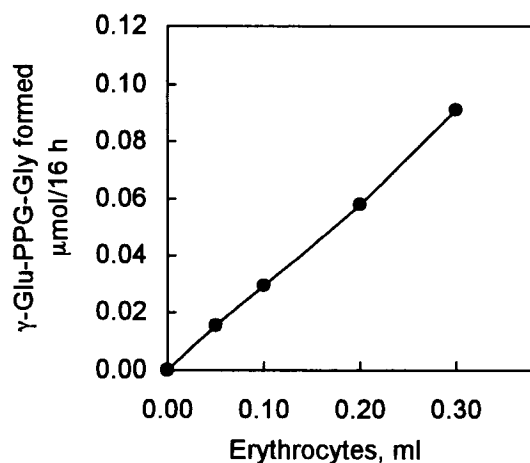
Substrate (mM)	$\gamma$ -Glu-PPG-Gly formed $\mu\text{mol/ml}$ of blood/16 h
D,L-Propargylglycine (2)	$0.036 \pm 0.009^b$
D,L-Propargylglycine (2) L-Glutamate (10) Glycine (10)	$0.094 \pm 0.013^c$
D,L-Propargylglycine (2) L-Glutamate (20) Glycine (20)	$0.172 \pm 0.028^d$

<sup>a</sup>: Substrates were incubated with 0.6 ml of human blood in a final volume of 0.8 ml for 16 h at 37°C and  $\gamma$ -Glu-PPG-Gly formed was determined as described under Materials and Methods. Values are expressed as mean  $\pm$  SD obtained from 4 separate experiments.

<sup>b</sup>, <sup>c</sup>, <sup>d</sup>: These superscript letters show the results of multiple comparison as follows: Values within a column and not followed by the same superscript letter are significantly different ( $P < 0.01$ ) by Tukey's  $\omega$ -procedure following one way analysis of variance.



**Fig. 2** Relationship between  $\gamma$ -glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) formation from D,L-propargylglycine in human erythrocytes and incubation time. Human erythrocytes, 0.3ml, were incubated with 2mM D,L-propargylglycine in the presence of 10mM L-glutamate and 10mM glycine in a final volume of 0.8ml for the indicated time at 37°C and  $\gamma$ -Glu-PPG-Gly formed was determined as described under Materials and Methods. Values are the means of 2 separate experiments.



**Fig. 3** Relationship between  $\gamma$ -glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) formation from D,L-propargylglycine in human erythrocytes and the amount of erythrocytes. D,L-Propargylglycine, 2mM, was incubated with the indicated amount of human erythrocytes in the presence of 10mM L-glutamate and 10mM glycine in a final volume of 0.8ml for 16h at 37°C and  $\gamma$ -Glu-PPG-Gly formed was determined as described under Materials and Methods. Values are the means of 2 separate experiments.

glycine may be actively metabolized to  $\gamma$ -Glu-PPG-Gly. The rate of  $\gamma$ -Glu-PPG-Gly formation for substrate

mixture without glutamate was significantly higher than that for substrate mixture without glycine, which could be explained by previously published results which indicate that the permeability of human erythrocytes to L-glutamate is limited (24).

Table 5 shows the relationship between D,L-propargylglycine concentration and  $\gamma$ -Glu-PPG-Gly formation in human erythrocytes in the presence of 10mM L-glutamate and 10mM glycine, which exhibits Michaelis-

**Table 2** Distribution of  $\gamma$ -glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) formed in human erythrocytes between the incubation medium and erythrocytes<sup>a</sup>

Compartment	$\gamma$ -Glu-PPG-Gly $\mu$ mol/ml of erythrocytes/16h (%)	
Total	0.322 $\pm$ 0.060	(100)
Erythrocytes	0.314 $\pm$ 0.058	(98 $\pm$ 4)
Incubation medium	0.008 $\pm$ 0.015	(2 $\pm$ 4)

<sup>a</sup>: Erythrocytes, 0.3ml, were incubated with 2mM D,L-propargylglycine in the presence of 10mM L-glutamate and 10mM glycine in a final volume of 0.8ml for 16h at 37°C. Two incubations were conducted for each erythrocyte preparation, and  $\gamma$ -Glu-PPG-Gly contents of the incubation mixture (total) and that of the incubation medium were determined as described under Materials and Methods.  $\gamma$ -Glu-PPG-Gly content of the erythrocytes was calculated by subtraction of the  $\gamma$ -Glu-PPG-Gly content of the incubation medium from that of the incubation mixture. Results are expressed as mean  $\pm$  SD obtained from 4 separate experiments. Values in parentheses indicate the percentage of the value of the 'total'.

**Table 3** Effect of freezing and thawing of erythrocytes on  $\gamma$ -glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) formation from D,L-propargylglycine in human erythrocytes<sup>a</sup>

Erythrocyte preparation	$\gamma$ -Glu-PPG-Gly formed $\mu$ mol/ml of erythrocytes/16h (%)	
Intact erythrocytes	0.320 $\pm$ 0.060	(100)
Frozen and thawed erythrocytes	0.007 $\pm$ 0.009	(2 $\pm$ 2)

<sup>a</sup>: Intact or frozen-thawed erythrocytes, 0.3ml, were incubated with 2mM D,L-propargylglycine in the presence of 10mM L-glutamate and 10mM glycine in a final volume of 0.8ml for 16h at 37°C and  $\gamma$ -Glu-PPG-Gly formed was determined as described under Materials and Methods. Results are expressed as mean  $\pm$  SD obtained from 4 separate experiments. Values in parentheses indicate the percentage of the value for intact erythrocytes.

**Table 4** Effect of extracellular glutamate and glycine on  $\gamma$ -Glu-PPG-Gly formation from D,L-propargylglycine in human erythrocytes<sup>a</sup>

Substrate mixture	$\gamma$ -Glu-PPG-Gly formed $\mu\text{mol/ml}$ of erythrocytes/16h (%)
Complete	$0.322 \pm 0.061^b$ (100)
Complete - Glu	$0.195 \pm 0.053^c$ ( $62 \pm 12$ )
Complete - Gly	$0.053 \pm 0.028^d$ ( $17 \pm 8$ )

<sup>a</sup>: Erythrocytes, 0.3ml, were incubated with substrate mixture in a final volume of 0.8ml for 16h at 37°C and  $\gamma$ -Glu-PPG-Gly formed was determined as described under Materials and Methods. The name of substrate mixture and the final concentrations generated by the mixture are as follows: complete, 2mM D,L-propargylglycine + 10mM L-glutamate + 10mM glycine; complete - Glu, 2mM D,L-propargylglycine + 10mM glycine; complete - Gly, 2mM D,L-propargylglycine + 10mM L-glutamate. Results are expressed as mean  $\pm$  SD obtained from 4 separate experiments. Values in parentheses indicate the percentage of the value for the substrate mixture, 'complete'. The  $\gamma$ -Glu-PPG-Gly formation observed for each of 3 different substrate mixtures used was significant by the Student's *t*-test ( $P < 0.05$ ).

<sup>b</sup>, <sup>c</sup>, <sup>d</sup>: These superscript letters show the results of multiple comparison as follows: Values within a column and not followed by the same superscript letter are significantly different ( $P < 0.01$ ) by Tukey's  $\omega$ -procedure following two way analysis of variance (factor 1, erythrocyte preparation; factor 2, substrate mixture).

**Menten kinetics.** From Lineweaver-Burk plots of these results, the  $K_m$  and  $V_{max}$  were calculated to be  $0.32 \pm 0.08\text{mM}$  and  $0.391 \pm 0.062\mu\text{mol/ml}$  of erythrocytes/16h ( $n = 5$ ), respectively. More than 28mg of propargylglycine per kg of body weight has often been administered in previous studies using rats or mice, where blood plasma propargylglycine level after the administration could be expected to be more than 0.25mM, assuming that the propargylglycine administered is distributed evenly throughout the whole body. The fact that the present  $K_m$  value is as low as 0.3mM is of interest when we consider *in vivo*  $\gamma$ -Glu-PPG-Gly formation in propargylglycine-administered animals or human subjects.

$\gamma$ -Glu-PPG-Gly is a major intermediate of propargylglycine metabolism in rat liver (21). The propargylglycine moiety of  $\gamma$ -Glu-PPG-Gly present in rat liver at 8h after the intraperitoneal administration of 50mg of D,L-propargylglycine per kg of body weight accounted for 8% of the total amount of D,L-propargylglycine administered (calculated from the data in our previous study (21)). The present results clearly show that  $\gamma$ -Glu-PPG-Gly is also formed as a metabolite of propargylglycine in human blood and erythrocytes. Because of the resemblance

between glutathione and  $\gamma$ -Glu-PPG-Gly,  $\gamma$ -Glu-PPG-Gly is thought to be formed by the same pathway as glutathione in human blood and erythrocytes as was hypothesized for  $\gamma$ -Glu-PPG-Gly formation in rat liver (21). It has been reported that 0.7% of the human erythrocyte glutathione pool ( $3.3\mu\text{mol/ml}$  of human erythrocytes) is synthesized per h in the presence of 0.25mM L-cystine, 1mM L-glutamate, and 0.5mM glycine (25), indicating that  $0.366\mu\text{mol}$  of glutathione per ml of erythrocytes is synthesized in a 16h period. It has also been reported that, when human erythrocytes were incubated with 30mM each of cysteine, glutamate, and glycine for 3h,  $0.16\mu\text{mol}$  of glutathione per ml of erythrocytes was formed (26). In comparison with these rates of glutathione synthesis in human erythrocytes, the present rate of  $\gamma$ -Glu-PPG-Gly formation is substantial. Considering the fact that glutathione is synthesized actively in various tissues, it is likely that metabolism of propargylglycine to  $\gamma$ -Glu-PPG-Gly may function as a major route of propargylglycine metabolism. Thus, the rate of  $\gamma$ -Glu-PPG-Gly formation and the  $K_m$  value for propargylglycine determined in the present study seem to indicate that metabolism of propargylglycine to  $\gamma$ -Glu-PPG-Gly can play a definite biological role, possibly to modulate the *in vivo* effect of propargylglycine, in human subjects who are loaded with propargylglycine.

**Table 5** Relationship between D,L-propargylglycine concentration and  $\gamma$ -glutamylpropargylglycylglycine ( $\gamma$ -Glu-PPG-Gly) formation in human erythrocytes<sup>a</sup>

D,L-Propargylglycine concentration, mM	$\gamma$ -Glu-PPG-Gly formed $\mu\text{mol/ml}$ of erythrocytes/16h (%)
0.2	$0.181 \pm 0.019$ ( $47 \pm 7$ )
0.4	$0.243 \pm 0.047$ ( $69 \pm 9$ )
1	$0.313 \pm 0.043$ ( $89 \pm 6$ )
2	$0.336 \pm 0.065$ (100)
4	$0.367 \pm 0.069$ ( $110 \pm 4$ )
10	$0.336 \pm 0.109$ ( $111 \pm 10$ )
20	$0.330 \pm 0.078$ ( $110 \pm 0$ )

<sup>a</sup>: Erythrocytes, 0.3ml, were incubated with the indicated concentration of D,L-propargylglycine in the presence of 10mM L-glutamate and 10mM glycine in a final volume of 0.8ml for 16h at 37°C and  $\gamma$ -Glu-PPG-Gly formed was determined as described under Materials and Methods. Results are expressed as mean  $\pm$  SD. The numbers of experiments are as follows: 0.2mM, 3; 0.4 to 4mM, 5 or 6; 10mM and 20mM, 2. Values in parentheses indicate the percentage of the value for incubation at 2mM D,L-propargylglycine which was conducted for all the erythrocyte preparations used.



It remains to be established how the metabolism of propargylglycine to  $\gamma$ -Glu-PPG-Gly is involved in the *in vivo* effect of propargylglycine. It could be expected that this metabolism of propargylglycine may lessen elevation of propargylglycine levels in tissues after loading with propargylglycine, diminishing the effect of propargylglycine itself. Further, because of the structural resemblance between glutathione and  $\gamma$ -Glu-PPG-Gly, there is the possibility that propargylglycine and  $\gamma$ -Glu-PPG-Gly may compete *in vivo* with cysteine and glutathione, respectively, in sulfur amino-acid metabolism (21). This possibility is of general interest since glutathione is involved in the metabolism of xenobiotics and peroxides. In the present study, we showed that  $\gamma$ -Glu-PPG-Gly can be synthesized in human erythrocytes and that erythrocytes containing approximately  $0.3\mu\text{mol}$  of  $\gamma$ -Glu-PPG-Gly per ml of erythrocytes can be prepared. Such a cellular system would be useful for studies concerning metabolism of propargylglycine to  $\gamma$ -Glu-PPG-Gly and its biological importance.

**Acknowledgments.** The authors wish to thank Mr. Okamoto and Mr. Iwasa of the Central Research Laboratory, Okayama University Medical School, for measurements of FAB mass spectrum.

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Received September 11, 1998; accepted October 26, 1998.